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TITLE: **CHEMICAL INHIBITORS OF SOLUBLE ADENYLYL CYCLASE (sAC)**

INVENTORS: **Jochen Buck and Lonny R. Levin**

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PROVISIONAL PATENT APPLICATION

Bicarbonate responsive 'soluble' adenylyl cyclase defines a nuclear cAMP microdomain

Introduction

Cyclic AMP is a nearly ubiquitous second messenger molecule that affects a multitude of cellular functions. In mammalian cells, two classes of adenylyl cyclase generate cAMP. Transmembrane adenylyl cyclases (tmACs) are tethered to the plasma membrane and regulated by heterotrimeric G proteins in response to hormonal stimuli [reviewed in (Hanoune and Defer, 2001)]. A second source of cAMP, the more recently described 'soluble' adenylyl cyclase (sAC), resides in discrete compartments throughout the cell (Zippin et al., 2003) and is regulated by the intracellular signaling molecules bicarbonate (Chen et al., 2000) and calcium (Jaiswal and Conti, 2003; Litvin et al., 2003).

Cyclic AMP elicits its cellular effects by activation of three known classes of effector proteins; Exchange Proteins Activated by cAMP (EPAC), Cyclic Nucleotide Gated Ion Channels (cNGC), and Protein Kinase A (PKA). A subset of these targets reside at the plasma membrane, where they exist in macromolecular signaling complexes which also include a receptor [a G protein coupled receptor (GPCR)], its transducing G-protein, and the source of cAMP, a tmAC isoform (Davare et al., 2001). The cAMP generated by tmACs acts locally (Rich et al., 2000; Rich et al., 2001; Zaccolo and Pozzan, 2002), most likely restricted by phosphodiesterase (PDE) 'firewalls' (Zaccolo and Pozzan, 2002) which define the limits of these cAMP signaling microdomains. But targets of cAMP do not solely reside at the plasma membrane. EPAC is localized to the nuclear membrane and mitochondria (Qiao et al., 2002), and PKA is tethered throughout the cell by a class of proteins called A-kinase anchoring proteins (AKAP) (Michel and Scott, 2002). The observation that cAMP does not diffuse

far from tmACs (Bacskai et al., 1993; Zaccolo and Pozzan, 2002) reveals there must be another source of cAMP modulating the activity of these distally localized targets.

'Soluble' adenylyl cyclase (Buck et al., 1999) is widely expressed in mammalian cells (Sinclair et al., 2000). Unlike tmACs, sAC is G protein insensitive (Buck et al., 1999), and among mammalian cyclases, it is uniquely responsive to intracellular levels of bicarbonate (Chen et al., 2000). The ubiquitous presence of carbonic anhydrases ensures that the intracellular bicarbonate concentration (and sAC activity) will reflect changes in pH (Pastor-Soler et al., 2003) and/or CO_2. Because CO_2 is the end product of energy producing metabolic processes, sAC is poised to function as a cell's intrinsic sensor of metabolic activity (Zippin et al., 2001). sAC possesses no transmembrane spanning domains (Buck et al., 1999) and is distributed to subcellular compartments containing cAMP targets (Zippin et al., 2003) which are distant from the plasma membrane. sAC was also found localized inside the mammalian cell nucleus (Zippin et al., 2003).

To evaluate how sAC-generated cAMP might differ from the second messenger generated by tmAC's, we explored a prototypical cAMP dependent pathway, PKA dependent phosphorylation of cAMP response element (CRE) Binding protein (CREB) (De Cesare and Sassone-Corsi, 2000). In a widely accepted signal transduction paradigm, extracellular signals (i.e., hormones and neurotransmitters) affect CREB family phosphorylation by stimulation of plasma membrane bound tmACs. The generated cAMP activates nearby PKA, and the liberated catalytic subunit then appears to translocate through the cytoplasm to phosphorylate and activate CREB proteins residing inside the nucleus (Hagiwara et al., 1993; Riabowol et al., 1988b). Intracellular signals, such as metabolic activity, also modulate CREB phosphorylation in a cAMP-

dependent manner (Daniel et al., 1998; Singh et al., 2001; Trumper et al., 2002), but the mechanism has yet to be established. Localization of sAC inside the nucleus, in close proximity to the CREB family proteins, and its regulation by calcium and bicarbonate, suggested that sAC might be responsible for modulating CREB activity in response to intracellular signals.

In this report, we demonstrate the existence of a nuclear cAMP signaling microdomain that mediates bicarbonate dependent activation of the transcription factor CREB. Bicarbonate activation of CREB represents an example of a mammalian cAMP dependent pathway solely modulated by intrinsic cellular signals. This nuclear cAMP signaling cascade functions independently from the classically defined mechanisms leading to CREB activation, demonstrating that cAMP is a locally acting second messenger which can work autonomously in different compartments within a single cell.

Results

Bicarbonate induces CREB phosphorylation

Bicarbonate treatment of cells uniquely activates sAC (Chen et al., 2000) while activation by G_s proteins or forskolin only stimulates tmACs; therefore, these agents can be used to differentially stimulate the two classes of mammalian adenylyl cyclase. To determine whether sAC activation would elicit PKA activation of CREB, a well-characterized target of tmAC generated cAMP, we treated cells with bicarbonate and measured PKA dependent phosphorylation of CREB using antisera specific for the PKA (Ser133) phosphorylated form of CREB. Hormonal stimulation of CREB transcription factors, acting through tmACs, reaches its peak in 30 minutes (Hagiwara et al., 1992). Treatment of COS7 cells with forskolin, which will activate the total cellular pool of tmACs, stimulated nuclear immunofluorescent staining and Western blot immunoreactivity using the phospho-specific antisera (Figures 1A,B). Treatment of COS7 cells for the same amount of time (30 minutes) with bicarbonate also resulted in CREB phosphorylation (Figures 1A,C). These increases in phospho-CREB immunostaining were inhibited by pretreatment with H89, confirming the involvement of PKA (Figures 1A-C). Overexpression of sAC led to an increase in basal CREB phosphorylation (Figure 1B+1C, lane 4) suggesting that sAC-generated cAMP was sufficient to activate CREB. Consistent with its bicarbonate responsiveness (Chen et al., 2000), sAC overexpressing COS7 cells displayed enhanced bicarbonate-dependent CREB phosphorylation (Figure 1C, lanes 2 and 5) which was also blocked by H89 (Figure 1C). The ability of either bicarbonate or forskolin to induce CREB phosphorylation reveals that CREB represents a downstream target of both tmAC and sAC generated cAMP.

Time course of bicarbonate induced CREB phosphorylation

We directly compared the time course of CREB activation in response to a hormonal activator of tmACs, PGE_2, versus the specific sAC activator, bicarbonate, in a liver cell line (Hagiwara et al., 1992). Phosphorylation of CRE binding proteins in response to bicarbonate occurred rapidly; increases in phospho-CREB were detected within 2 minutes, the earliest time tested (Figure 2A). In contrast, PGE_2 (Figure 2B) or forskolin (data not shown) stimulation of CREB phosphorylation was detectable only after 5 minutes, consistent with published reports (Hagiwara et al., 1992). The longer activation kinetics following PGE_2 or forskolin stimulation is thought to reflect the time required for translocation of PKA catalytic subunit into the nucleus from the plasma membrane where it was activated by a hormonally modulated tmAC (Hagiwara et al., 1993). In addition to being more rapid, the peak intensity of phosphorylation was higher with bicarbonate treatment. The different kinetics and intensity of CREB activation by bicarbonate and PGE_2 reveal that while sAC and tmACs may affect overlapping substrates, they may participate in distinct signal transduction cascades.

CREB, sAC, and PKA co-exist in the nucleus

Because CREB family members and sAC (Zippin et al., 2003) reside inside the nucleus, we reasoned the accelerated kinetics and intensity of bicarbonate-induced CREB activation could occur if sAC and CREB co-existed in a signal transducing complex. A complete nuclear cAMP signaling cascade capable of phosphorylating CREB family proteins requires the presence of the cAMP-responsive PKA holoenzyme. Both catalytic and regulatory subunits of PKA have been immunologically (Jungmann

et al., 1988; Kuettel et al., 1985; Yang et al., 1998) and biochemically (Byus and Fletcher, 1982; Constantinescu et al., 1999; Murray et al., 1985; Zhang et al., 1996) detected inside the nucleus, and nuclear localization of PKA holoenzyme has been described in lower eukaryotes (Griffioen et al., 2000), but the nuclear presence of PKA regulatory subunit, and therefore cAMP-responsive holoenzyme, has been questioned. We repeated and extended the immunological examination of regulatory subunit localization and confirmed that PKA resides inside the nucleus of the human liver cell line, Huh7 (Figure 3A), suspension HeLa cells (Figure 3B-C), and in a subset of cells within sectioned liver tissue (Figure 4A). Confocal microscopy of Huh7 and HeLa cells using polyclonal and monoclonal antibodies, recognizing PKA regulatory subunit isoforms (RIα, RIIα), revealed distinctive cytoplasmic staining in accordance with accepted dogma (Alto et al., 2002) (Figure 3A-C), but these regulatory subunit isoforms were also detected inside the nucleus (Figure 3A-C and 4A). In the case of suspension HeLa cells, it should be stressed that these optical slices were selected to illustrate the intranuclear staining of PKA. Slide preparation and imaging constraints cause PKA cytoplasmic staining to appear as a thin layer surrounding the nucleus (Figure 3B-C, arrows marked A) and within the expanse of cytoplasm stretching out as these suspension cells adhere to the coverslip (Figure 3B-C, arrows marked B).

Nuclear staining of each isoform was distinct. RIIα was present in a diffuse pattern throughout the nucleus with small areas of enrichment (Figure 3A, middle panels and 3B), whereas RIα was distributed in the nucleoplasm but more enriched in nucleoli (Figure 3A, right panels and 3C). RIIα was also detected in the nuclei of a subset of rat liver primary hepatocytes (Figure 4A). Consistent with our previously published data (Zippin et al., 2003), sAC was also present in the nuclei of Huh7 cells

(Figure 3D) and a subset of rat liver hepatocytes (Figure 4). PKA, sAC, and phosphorylated CREB seem to be co-ordinately localized; the subset of nuclei in rat liver hepatocytes and Huh7 cells (data not shown) positive for sAC protein (Figure 4A and B, arrow A) also contained R subunit (Figure 4A, arrow A) and CREB phosphorylation (Figure 4B, arrow A), whereas nuclei not enriched for sAC displayed neither R subunit nor CREB phosphorylation (Figure 4A and B, arrow B). Rat liver hepatocytes positive for sAC, PKA, and phospho-CREB represent approximately 10% of total hepatocytes, and we have not yet identified any consistency with known liver anatomy. These data demonstrate that nuclei contain all the components of a cAMP signalling cascade and suggest that sAC generated cAMP is positioned to activate nuclear PKA holoenzymes to phosphorylate CREB proteins.

Isolated nuclei contain components of a cAMP signaling microdomain

Bicarbonate treatment of whole cells leads to rapid induction of CREB phosphorylation (Fig. 2). To test whether the nuclear localized sAC and PKA were responsible for this bicarbonate induced CREB activation, we prepared isolated nuclei from suspension HeLa cells, a cell line with well established protocols for the isolation and enrichment of nuclei. Cells were lysed using digitonin and nuclear preparations were purified by density centrifugation through an Optiprep gradient. Western analyses of the same cell equivalents from each fraction using cellular markers for different subcellular compartments (histone H3, Na/K ATPase α1 subunit, cytochrome c oxidase subunit III (COX), and β–tubulin) confirmed that the nuclear fractions (P2) were positive for nuclear markers (histone) with undetectable levels of plasma membrane (Na/K ATPase), mitochondrial (COX), or cytoplasmic (tubulin) contamination (Figure

5A). To confirm that the P2 fraction did not contain any detectable mitochondria, a possible source of both sAC and PKA contamination, we overloaded the P2 fraction, but COX antigen was still not detected (data not shown). Visual inspection and DAPI fluorescence confirmed that the final preparation was enriched for intact nuclei (Figures 5B and 5C), and as expected, isolated nuclei contained both CREB and sAC proteins by immunocytochemistry (Figure 5B) and by Western blotting (Figure 5D)

Consistent with staining patterns shown above (Figures 3A-C), RIIα immunostaining was present throughout the nucleus, while RIα appeared enriched within the nucleolus (Figure 5C). PKA RIα and RIIα were also detected by Western analysis as a single band of the predicted molecular weight in the P2 lysate, using monoclonal and polyclonal antibodies, (Figure 5E) confirming the specificities of these antibodies for immunostaining. Because the staining patterns of isolated HeLa cell nuclei (Fig. 5C) reflected the immunostaining pattern observed in intact HeLa cells (Fig. 5B-C), we concluded that the isolation and enrichment of nuclei had little effect on nucleoplasm architecture.

sAC represents the only source of cAMP detectable in isolated nuclei

We previously demonstrated that sAC activity was present in COS7 cell nuclei (Zippin et al., 2003). We now show that bicarbonate responsive sAC is the only source of cAMP in nuclei isolated from suspension HeLa cells. Whereas forskolin potently stimulates cAMP production in whole cell lysates (Figure 6A), there was no significant increase in cAMP elicited by forskolin in isolated nuclei (Figure 6B). There was a significant level of basal adenylyl cyclase activity in isolated nuclei, which was stimulated by bicarbonate addition (Figure 6B). Both the bicarbonate stimulated and

basal activities were inhibited by sAC-selective inhibitors (Figure 6C,D). We have identified several sAC inhibitors (Figure 6C), inert towards tmACs (Figure 6D), in a screen of a combinatorial chemical library (K. H., T.N. Litvin, E. Hyde, J.H.Z., L.R.L., and J.B., in preparation). In the presence of two representative, structurally unrelated inhibitors (KH1 and KH2 each display an IC_{50} of ≤ 10 μM towards recombinant human sAC protein), the cAMP generated in the presence of bicarbonate in P2 nuclei was reduced to a level below that of basal. These results indicate that in addition to mediating the bicarbonate-induced increase in cAMP in isolated nuclei, sAC is also responsible for the observed basal adenylyl cyclase activity.

Bicarbonate induces CREB phosphorylation in isolated nuclei

CREB phosphorylation in isolated nuclei was assayed by immunocytochemistry using phospho-CREB specific antisera (Figure 7A-B). Nuclei incubated in the presence of either bicarbonate or cAMP displayed at least a two-fold rise in the percentage of phospho-CREB positive nuclei relative to untreated nuclei (basal) (Figure 7B). As expected, due to the lack of tmACs in isolated nuclei, the number of nuclei positive for CREB phosphorylation was unaffected by forskolin. These data demonstrate that a bicarbonate-responsive signaling cascade leading to CREB phosphorylation is wholly contained within the mammalian cell nucleus. In contrast, the hormone and forskolin-responsive tmAC defined cascade is only functional in a whole cell context.

Nuclear sAC activates CREB via nuclear PKA

To facilitate the use of pharmacological reagents to further evaluate bicarbonate-induced CREB phosphorylation, we monitored CREB phosphorylation by Western analysis (Figure 7C-D). Similar to our observations using immunocytochemistry, treatment of isolated nuclei with bicarbonate or 8-Br-cAMP elicited a 27-fold or 30-fold increase in CREB phosphorylation, respectively (Figure 7C). Once again, forskolin, which had a potent effect in a whole cell context (Figure 1B), elicited no significant stimulation of CREB phosphorylation in isolated nuclei (Figure 7D).

We next confirmed that the effects of bicarbonate on CREB phosphorylation were mediated by nuclear sAC and PKA. CREB phosphorylation induced by bicarbonate was substantially reduced by the PKA inhibitors, H89 (50%) and RpcAMPs (70%) (Figure 7C), revealing the involvement of cAMP-responsive PKA holoenzyme. The chemical inhibitors effective at blocking sAC generated cAMP accumulation (KH1 and KH2, Figure 6B) were also effective at preventing bicarbonate induced CREB phosphorylation (Figure 7D) demonstrating, once again, that sAC is responsible for the bicarbonate-stimulated cAMP-dependent, phosphorylation of CREB in the mammalian cell nucleus.

Discussion

Most cellular pathways in eukaryotic cells are impacted by cAMP. Effectors of cAMP mediate processes at both the plasma membrane and multiple, distinct intracellular sites. It has been widely assumed that cAMP is generated exclusively at the plasma membrane by G protein-regulated tmACs, and the second messenger then diffuses from the cell membrane through the cytosol to its intracellular targets. However, FRET-based (Bacskai et al., 1993; Zaccolo and Pozzan, 2002) and biochemical (Rich et al., 2000; Rich et al., 2001) methods for observing intracellular cAMP concentrations reveal that the second messenger generated by tmACs does not diffuse far from its site of synthesis. We have recently demonstrated that sAC is localized at multiple, subcellular compartments throughout the cell, including mitochondria, centrioles, the mitotic spindle, the mid-body and the nucleus (Zippin et al., 2003), each of which contain targets of cAMP. These data suggest that the cell may contain multiple, independently modulated cAMP signaling microdomains; targets near the plasma membrane would depend upon tmACs for second messenger generation, while targets inside the cell would be modulated by sAC generated cAMP (Wuttke et al., 2001; Zippin et al., 2003). We now provide data supporting this hypothesis by demonstrating the existence of a sAC-defined nuclear cAMP signalling microdomain which can lead to CREB activation.

The nuclear cAMP signaling cascade induced by bicarbonate produced a rapid activation of CREB family members in both whole cells and nuclei, while PGE_2 and forskolin, tmAC specific activators, produced a delayed response exclusively in whole cells. Therefore, cAMP-mediated activation of CREB family members by tmACs and sAC proceed via independent pathways. CREB activation by hormones or

neurotransmitters via tmACs apparently requires time for movement of PKA catalytic subunit from the plasma membrane into the nucleus (Hagiwara et al., 1993; Riabowol et al., 1988a). This delayed activation is consistent with hormonal control of gene expression providing a long-term response to predominantly sustained extracellular signals (Bailey et al., 1996). In contrast, the newly described nuclear sAC activation pathway proceeds rapidly without requiring the translocation of any constituent. In this regard, the sAC nuclear microdomain is capable of responding quickly to subtle fluctuations in intrinsic signals, such as local intracellular concentrations of bicarbonate and calcium.

In tissues, sAC is not present within the nucleus of every cell. In liver, sAC appears to be predominantly extranuclear but enriched in a subset of the nuclei (Figure 4, arrows A). PKA holoenzyme appears to be enriched within the same subset of nuclei (Figure 4A, arrow A), and interestingly, these are the nuclei which are also positive for CREB phosphorylation (Figure 4B, arrow A). The presence of both positive and negative nuclei for sAC, PKA, and CREB phosphorylation in the same tissue suggests that there may be coordinated regulation of the presence of this newly described nuclear signaling microdomain.

The demonstration that bicarbonate treatment of whole cells leads to activation of the CREB family of transcription factors reveals that bicarbonate itself induces a signal transduction cascade. Cellular bicarbonate levels reflect intracellular pH as well as CO_2 generation (Bevensee et al., 2000); therefore, bicarbonate signaling pathways would respond to a wide variety of cellular transitions. Immunostaining revealed that sAC is present at mitochondria, centrioles, mitotic spindles, and mid-bodies (Zippin et al., 2003), suggesting the existence of multiple cAMP signaling microdomains within a

single cell. A remaining challenge will be to determine whether sAC molecules in these different microdomains are subject to independent and unique modes of regulation, permitting a variety of distinct responses independently mediated by the same second messenger.

Materials and Methods

Cell Growth and Transfections

All cell lines were grown in DMEM (44 mM sodium bicarbonate) supplemented with 10% FBS. Where indicated, cells were transfected using Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. Cells were incubated with DNA for 5 hours in OPTI-MEM and then switched to normal media. Bicarbonate starvation was conducted by changing media to bicarbonate-free DMEM (44 mM Hepes) supplemented with 10% FBS for at least 1 hour at 37°C under ambient air conditions. Bicarbonate stimulation consisted of returning cells to normal bicarbonate containing media and placing them in a 5% CO_2 incubator. For PGE_2 or forskolin stimulation, cells were grown in normal media under 5% CO_2. Stimulation was accomplished by replacing media with normal media containing 1 μM PGE_2 or 10μM forskolin. For Western analysis, cells were lysed immediately by direct addition of SDS sample buffer.

Immunocytochemistry

Cells or nuclei were washed in PBS and fixed for either 30 minutes in 4% PFA and permeabilized in 0.1% Triton X-100 or fixed for 15 minutes in 2% PFA and permeabilized in 0.05% Triton X-100. Liver from adult rat was rapidly excised, placed between two thinly sliced pieces of bovine liver, and snap frozen in isopentane cooled with liquid nitrogen. Cryosections (6 μM thick) were collected on superfrost slides (Fisher Scientific) and stained within 1 day of sectioning. Tissue was fixed for 30 minutes in 4% PFA and permeabilized in 0.1% Triton X-100 for 15 minutes. All samples were blocked in 2% BSA for at least one hour. Cells or tissues were stained with anti-sAC R41 or R52 biotinylated monoclonal antibodies or R21 monoclonal

antibody (1:100) generated against human sAC_t antigen as previously described (Zippin et al., 2003), anti-PKA regulatory subunit (RIα and RIIα) polyclonal antisera (1:100, Chemicon International and Cedarlane Laboratories Limited) or monoclonal antibodies (Becton-Dickinson Transduction Laboratories), and anti-CREB or anti-phospho-CREB polyclonal antisera (1:500, Cell Signaling Technologies) overnight in 2% BSA, 0.01% Triton X-100, washed for 3 times ten minutes each in 2% BSA, 0.01% Triton X-100, stained for one hour at room temperature with goat-anti-rabbit Alexa Fluor 488, goat-anti-mouse Alexa Fluor 568, or goat-anti-mouse Alexa Fluor 594 (Molecular Probes), treated with DAPI for 5 minutes or To-Pro 3 (1:500, Molecular Probes) for 15 minutes, and then washed and mounted with gelvatol/DABCO (Sigma).

For phospho-CREB immunolocalization, cells or nuclei were fixed in 4% PFA for 30 minutes, permeabilized in 0.1% Triton X-100 for 15 minutes, blocked for at least one hour in 3% BSA, and immunostained using phospho-CREB polyclonal antisera (1:500, Cell Signaling Technologies) overnight at 4°C. Staining was visualized by incubation with goat-anti-rabbit Alexa Fluor 488 (Molecular Probes) for one hour at room temperature, treated with DAPI for 5 minutes, and then washed and mounted with gelvatol/DABCO (Sigma). Fluorescent images were recorded by a Hamamatsu digital camera connected to a Nikon inverted epifluorescent microscope. Images were taken at the same exposure time and gain, and all photographic manipulations were performed equally. Phospho-CREB positive nuclei were quantified in multiple fields from each stained slide by a blinded experimenter.

Confocal images were acquired with a Zeiss LSM 510 confocal system. Goat-anti-rabbit Alexa Fluor 488 was excited with a 488 nM Kr/Ar laser, goat-anti-mouse

Alexa Fluor 568 was excited with a 568 nm Kr/Ar Laser, and To-Pro 3 was excited with a 633 nm Kr/Ar Laser.

Isolation of Nuclei

Nuclei were isolated by cellular lysis followed by differential centrifugation (Spector et al., 1998) through OptiPrep (Axis-Shield). HeLa cells grown in suspension were lysed by detergent treatment in TM-2 buffer (0.01 M Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, 150 mM NaCl, 0.5 mM PMSF, 10 μg/ml Apoprotin, 10 μg/ml Leupeptin) containing 100 μg/ml digitonin followed by a 1000 x g spin. Supernatant (S1) was removed and the pellet was then resuspended in 0.25 M sucrose, 25 mM KCl, 30 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.8. The resuspended pellet and 60% OptiPrep iodixanol were mixed (30% Optiprep final) and centrifuged at 10,000 x g for 20 minutes. The supernatant was removed and the nuclei-enriched pellet (P2) was resuspended in TM-2 buffer without detergent.

CREB Phosphorylation and Adenylyl Cyclase Assays

Equal aliquots of nuclei-enriched P2 preparations were incubated in 50 μl final volume of 100 mM Tris, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP for CREB phosphorylation and 100 mM Tris, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM IBMX for adenylyl cyclase assay with the indicated additions for 10 minutes (CREB phosphorylation) or 15 minutes (adenylyl cyclase) at 37° C. Reactions were stopped by addition of 20 μL of SDS sample buffer (CREB phosphorylation) or by being placed into a 100° C heat block for 3 minutes (adenylyl cyclase).

For whole cell and isolated nuclei CREB phosphorylation assays, equal cell or nuclear equivalents were separated under reducing conditions using a 10% SDS-PAGE, transferred to a PVDF membrane, and probed for CREB (rabbit polyclonal antiserum, Upstate Biotech) and phosphorylated CREB (rabbit polyclonal antiserum, Upstate Biotech). HRP conjugated secondary antibodies were used and bands were visualized using enhanced chemiluminescence. Fluorchem 8800 image analysis software (Alpha Innotech) was used to quantitate Western results. Intensities of phospho-CREB bands were normalized to total CREB.

cAMP produced in the cyclase assays was detected using a competition based assay with ^{3}H-cAMP (Amersham Pharmacia) and compared to a cAMP standard curve for quantitation.

Inhibitor profiles were determined by adenylyl cyclase assay (Assay Designs, Inc.) using purified sAC protein (Litvin et al., 2003) in the presence of 10 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 mM ATP, or a mixture of purified catalytic domains, C1 and C2, from Type VII transmembrane adenylyl cyclase (Yan and Tang, 2002) in the presence of 5 mM $MgCl_2$, and 1 mM ATP as previously described.

Quantitation of isolated nuclei (P2 fraction) immunocytochemistry

Nuclei were treated with Mg^{2+}-ATP alone or in combination with bicarbonate, forskolin, or 8-Br-cAMP as indicated above for 10 minutes, spread on a chilled slide, stored at –20°C, and then immunostained using phospho-CREB specific antisera as described. Nuclei were also treated with DAPI to differentiate intact nuclei from membrane ghosts. DAPI positive nuclei were scored for phosho-CREB immunofluorescence. Nuclei with detectable staining (i.e., $NaHCO_3$ panel, Figure 7A)

were considered positive for CREB phosphorylation whereas nuclei with no detectable staining (i.e., basal panel, Figure 7A) were counted as negative. Multiple microscopic fields were photographed for each condition, and data was combined from three to five separate experiments.

Western Analysis

Equal cell equivalents, unless otherwise noted, were separated under reducing conditions using a 10% SDS-PAGE, transferred to PVDF membrane, and blocked in 5% Milk. The blots were then probed with antibodies against either NaK ATPase (monoclonal, 1:50, Santa Cruz), histone H1 (monoclonal, 1:100, Santa Cruz), cytochrome oxidase subunit III (monoclonal, 2μg/ml, Molecular Probes), β-tubulin (monoclonal, 1:1000, Sigma), sAC (R21 monoclonal antibody, 1:500), monoclonal RIα or RIIα antibodies (1:250, Becton Dickinson), or polyclonal RIα or RIIα antisera (1:5000, Chemicon) overnight. HRP conjugated secondary antibodies were used and bands were visualized using enhanced chemiluminescence.

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Abbreviations used in the paper:

AKAP, A kinase anchoring protein; cNGC, cyclic nucleotide gated channels; CRE, cAMP Response Element; CREB, CRE Binding Protein; DIC, differential interference contrast microscopy; EPAC, Exchange Protein Activated by cAMP; GPCR, G-protein coupled receptor; PDE, phosphodiesterase; PFA, paraformaldehyde; PKA, protein kinase A; sAC, soluble adenylyl cyclase; sAC_t, 48 kD isoform of sAC; tmAC, transmembrane adenylyl cyclase; Fsk, forskolin.

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Figure Legends

Figure 1. **Bicarbonate induces CREB phosphorylation via sAC activation in a PKA dependent manner.** (A) COS7 cells were starved for bicarbonate 60 minutes, and were either incubated in the same bicarbonate starvation media for an additional 30 minutes (no treatment); incubated in normal, bicarbonate-containing DMEM in 5% CO_2 for 30 minutes ($NaHCO_3$); or preincubated with H89 (10 μM) for 10 minutes followed by incubation in normal, bicarbonate-containing DMEM in 5% CO_2 for 30 minutes ($NaHCO_3$ + H89). As control, COS7 cells grown in normal DMEM in 5% CO_2 were incubated with 10 μM forskolin for 30 minutes (forskolin). Cells were then immunostained with phospho-CREB antisera (green). Phase images of cells on left. Bar = 50 μm. (B) COS7 cells were transfected with vector control or sAC_t, and cells were assayed 36 hours post transfection. Cells were treated with vehicle control (DMSO) or H89 (10 μM) for 10 minutes and then stimulated with forskolin (10 μM) or given vehicle control (DMSO) for an additional 30 minutes. (C) Transfected cells were starved for bicarbonate, and were either incubated in the same bicarbonate starvation media for an additional 30 minutes; incubated in normal, bicarbonate-containing DMEM in 5% CO_2 for 30 minutes; or preincubated with H89 (10 μM) for 10 minutes followed by incubation in normal, bicarbonate-containing DMEM in 5% CO_2 for 30 minutes. (B & C) Top panel is Western blot using anti-phospho-CREB antisera; middle panel is Western blot using CREB-specific antisera with Phosphorylated CREB (Ⓟ CREB) protein and total CREB indicated. Shown below are the intensities of phospho-CREB relative to CREB normalized to vector control no treatment (lane 1).

Figure 2. **Time course of CREB phosphorylation by sAC and tmAC.** (A) Huh7 cells were starved for 1 hour for bicarbonate and CO_2 and then incubated in normal, bicarbonate-containing (44 mM) DMEM in 5% CO_2 for the time indicated or (B) kept in normal media and treated with PGE2 (1 μM) for the time indicated. (A & B) Top panel is Western blot using anti-phospho-CREB antisera; middle panel is Western blot using CREB-specific antisera with Phosphorylated CREB (Ⓟ CREB) protein and total CREB indicated. Shown below are graphical representations of the intensities of phospho-CREB relative to CREB normalized to the 0 minute time point (lane 1).

Figure 3. **Immunocytochemistry detects both sAC and PKA in the mammalian cell nucleus.** (A) Confocal immunocytochemistry of Huh7 cells with monoclonal RIIα (center panels, green) and polyclonal RIα antibody (right panels, red). To-Pro 3 (upper left). Overlay of To-Pro 3 with both RIIα and RIα (lower left), RIIα (lower middle), and RIα (lower right). Secondary controls were negative (data not shown). (B-C) Confocal images of suspension HeLa cells immunostained with (B) PKA regulatory subunit RIIα polyclonal antisera and (C) PKA regulatory subunit RIα monoclonal antibody. Arrow A and B indicate suspension HeLa cytoplasm. Secondary controls were negative (insets). (D) Confocal immunocytochemistry of Huh7 cells stained with R41 monoclonal antibody against sAC.

Figure 4. **Activated CREB , sAC, and PKA are present within the same rat liver nuclei.** (A) Rat liver section stained with DAPI (DNA, blue, upper left), R52 biotinylated monoclonal antibody (sAC, green, upper middle), and polyclonal RIIα

antisera (RIIα, red, upper right), overlays of RIIα and sAC (lower left), sAC and DAPI (lower middle), and RIIα and DAPI (lower right). Arrows marked A indicate nuclei enriched for both sAC and PKA, whereas arrows marked B refer to nuclei not enriched for either. (B) Rat liver section stained with DAPI (DNA, blue, upper left), R21 monoclonal antibody (sAC, green, upper middle), and polyclonal P-CREB antisera (P-CREB, red, upper right), overlays of P-CREB and sAC (lower left), sAC and DAPI (lower middle), and P-CREB and DAPI (lower right). Arrows marked A indicate nuclei enriched for both sAC and P-CREB, whereas arrows marked B refer to nuclei enriched for neither. Rat liver tissue immunolocalization was confirmed to be inside the nucleus by confocal microscopy (data not shown).

Figure 5. **sAC, PKA, and CREB coexist in mammalian cell nuclei.** (A) Western blots of cell equivalents from HeLa whole cells (WC), low speed supernatant (S1), and nuclear-enriched high speed pellet (P2) probed with antibodies against NaK ATPase (NaK), histone H1 (Histone), cytochrome oxidase subunit III (COX), and β-tubulin (Tubulin). (B) Immunocytochemistry of nuclei isolated from HeLa cells (P2 pellet) using CREB polyclonal antisera (red) and sAC R52 biotinylated monoclonal antibody (green). DIC and DAPI (blue) images shown. Bar = 10 μm. (C) Nuclei isolated from HeLa cells (P2 pellet) immunostained with polyclonal antisera (green) and monoclonal antibodies (red) directed against both RIα and RIIα indicated that both proteins maintained their nucleoplasmic architecture throughout the fractionation procedure. Bottom row represents staining with goat-anti-rabbit (middle panel) or goat-anti-mouse controls (right panel) alone. Left column represents DAPI images in blue. Bar = 10 μm. (D) Western blot of nuclear enriched P2 for sAC with R21 monoclonal antibody. (E)

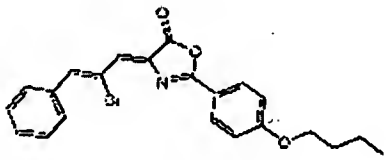
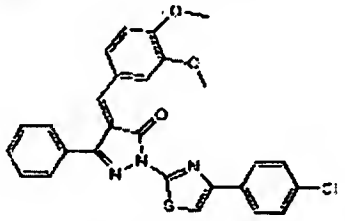
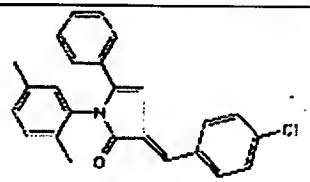
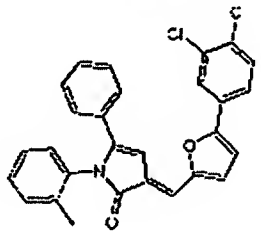
Western blots of nuclear cnriched P2 pellet with monoclonal (mRIα) and polyclonal (pRIα) antisera against RIα and with monoclonal (mRIIα) and polyclonal (pRIIα) antisera against RIIα. All Westerns blots resolved only single bands of the predicted molecular weights.

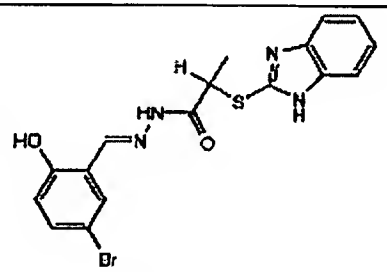
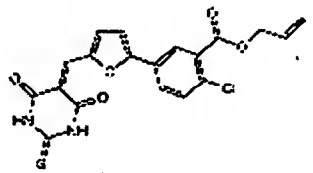
Figure 6. **sAC activity is the only detectable adenylyl cyclase activity in the mammalian cell nucleus.**

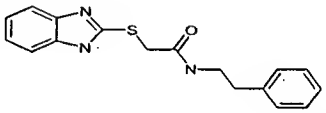
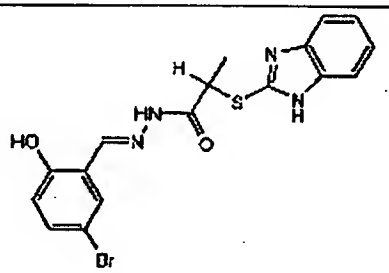
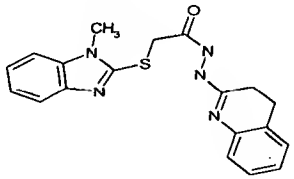
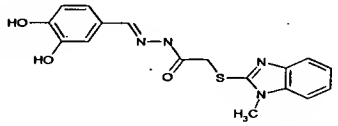
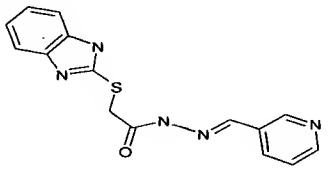
(A) Adenylyl cyclase assay of whole cell lysate with Mg^{2+}-ATP alone (Basal) or Mg^{2+}-ATP and forskolin (Fsk). (B) Cyclase assay of nuclear lysate with Mg^{2+}-ATP alone (Basal), or in the presence of 10 μM forskolin (Fsk), or 40 mM bicarbonate (+$NaHCO_3$) in the absence (-) or presence of the sAC specific inhibitors KH1 (250 μM) or KH2 (100 μM). cAMP values are expressed as pmoles produced per ml of lysate and represent averages of duplicate determinations with standard deviation about the means indicated. These data are representative of at least 3 independent experiments performed in duplicate. (C) Chemical structures of inhibitors KH1 and KH2. (D) Inhibition of purified sAC (circles) or purified tmAC type VII C1 + C2 (squares) by KH1 (open symbols) and KH2 (filled symbols). Values for purified tmAC type VII C1 + C2 represent basal activity which was significantly above background.

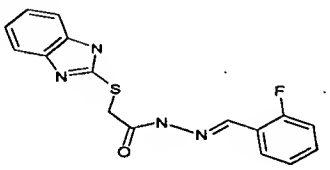
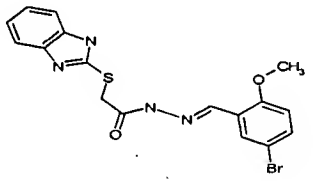
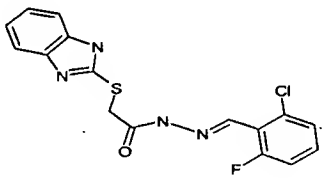
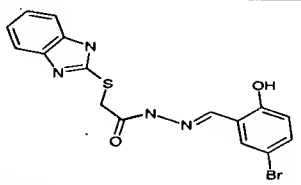
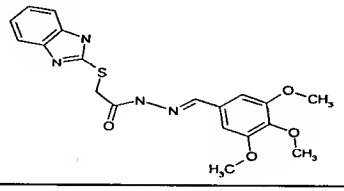
Figure 7. **Isolated nuclei contain a bicarbonate-responsive cAMP signaling microdomain dependent on both sAC and PKA.** (A-B) Equal aliquots of nuclei enriched P2 were incubated with 40 mM NaCl (Basal), 10 μM forskolin (FSK), or 40 mM sodium bicarbonate ($NaHCO_3$) for 10 minutes, smeared on a chilled glass slide, placed at –20°C, and immunostained for CREB family member phosphorylation using

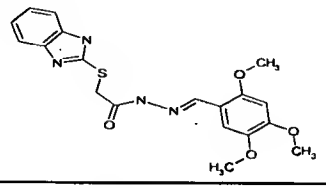
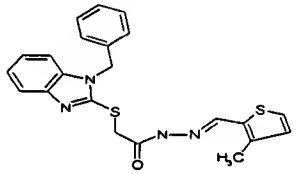
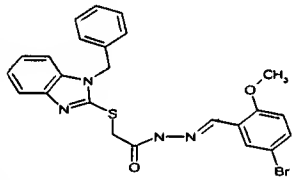
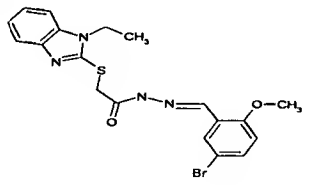
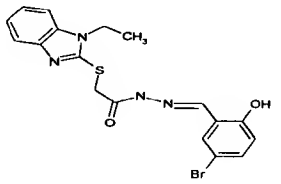
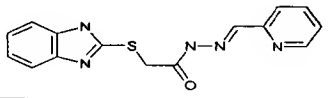
phospho-CREB-specific polyclonal antisera (green). (A) Intact nuclei were confirmed by DAPI staining (left panels). Lower right panel highlights representative nuclei considered positive for CREB phosphorylation for quantitation. (B) Three microscopic fields per condition were photographed and counted by a blinded scientist. Values graphed represent the percentage of positive nuclei normalized to Basal (control) averaged from five separate experiments. Ratios above each bar represent the total number of positive nuclei divided by the total number of nuclei counted for all five experiments. (C) Western blot using phospho-CREB specific antisera against equal aliquots of nuclei-enriched P2 treated with Mg^{2+}-ATP alone (Basal), or substrate in the presence of 1 mM 8-Br-cAMP (cAMP), 40 mM bicarbonate, or 40 mM bicarbonate in the presence of either 10 μM H-89 or 1 mM 8-Br-RpcAMPs. Each band was quantitated and normalized to basal; the relative intensities are basal (1 unit), cAMP (30 units), bicarbonate alone (27 units), bicarbonate plus H-89 (13 units), and bicarbonate plus Rp-cAMPs (8 units). (D) Western blot using phospho-CREB specific antisera against equal aliquots of nuclei-enriched P2 treated with 10 μM forskolin (FSK) or with 40mM bicarbonate alone or in the presence of either sAC specific inhibitor KH1 (250 μM) or KH2 (100 μM).

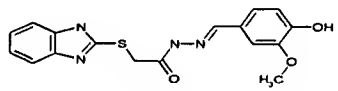
Nam	screen #	Structure	In vitro IC50				In vivo IC50		Assay results
			pure sAC	pure sol.AC7	sAC in extracts	tmAC in extracts	sAC (basal)	tmAC (FSK)	
KH1	0782-6838		23 μM	> 200 μM			>>	>>	inhibits nuclear sAC inhibits pCREB MAY activate at ≤1 μM
KH2	2425-4155		2 μM	> 400 μM	>>100	>>100	>>	>>	inhibits nuclear sAC inhibits pCREB
KH3	2150-0290		8 μM	> 500 μM	>>100	>>100	>>	>>	inhibits pCREB
KH4	2150-0028		6 μM	>> 27 μM	>>100	>>100	>>	>>	inhibits pCREB
KH5	1683-2649		>> 55 μM	>> 55 μM	10-50 μM	100 μM	100 μM	>>	inhibits nuclear sAC inhibits pCREB
KH6	K074-5853		20 μM	>> 12 μM					

KH7	**1185-0408**		**1 µM**	**>> 55 µM**	**10 µM**	**>>100**	**10 µM**	**> 500 µM**	**inhibits nuclear sAC inhibits pCREB**
KH8	2237-1900		0.5 µM	0.5 µM					
KH9									
KH10									

Nam	scr en #	Structure	In vitro EC50				In vivo IC50		Assay results
			pure sAC	pure tmAC	sAC in extracts	tmAC in extracts	sAC (basal)	tmAC (FSK)	
KH5	1683-2649		> 1 mM	>> 55 µM	10-100 µM	≥ 100 µM	100 µM	>>	inhibits nuclear sAC inhibits pCREB
KH7	**1185-0408**		**1 µM**	**>> 55 µM**	**< 10 µM**	**>> 100 µM**	**10 µM - 30 µM**	**> 500 µM**	**inhibits nuclear sAC inhibits pCREB Inhibits insulin secretion**
KH7.01	1185-0325		>100						
KH7.02	1185-0453		≥100				30 µM	≥200 µM	
KH7.03	2311-0174		≥300						

KH7.04	2311-0176		≥50						
KH7.05	2311-0184		~30				30 µM	>200 µM	
KH7.06	2311-0186		~30						
KH7.07	2311-0195		10				30 µM	>200 µM	
KH7.08	2311-0196		≥200				70 µM		

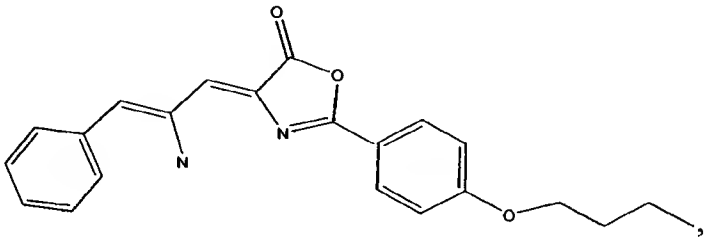
KH7.09	2311-0203		≥200						
KH7.10	2423-4824		80						
KH7.11	2423-4830		65						
KH7.12	2423-5085		30						
KH7.13	2423-5096		75						
KH7.14	3253-0230		>1mM				~200 µM		

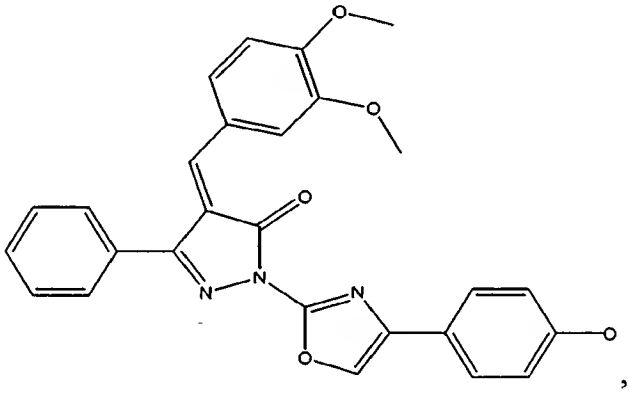
KH7.15	3253-0233		~1mM				>200 µM		INERT on Insulin secretion

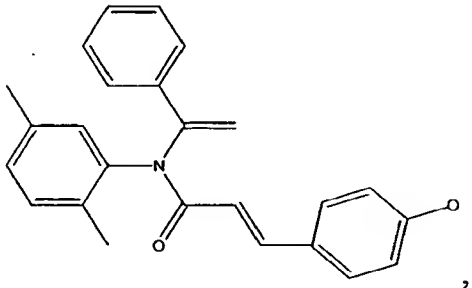
WHAT IS CLAIMED:

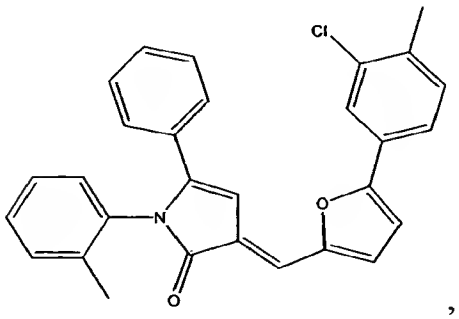
1. A method of treating a disorder in a subject mediated by soluble adenylyl cyclase, said method comprising:

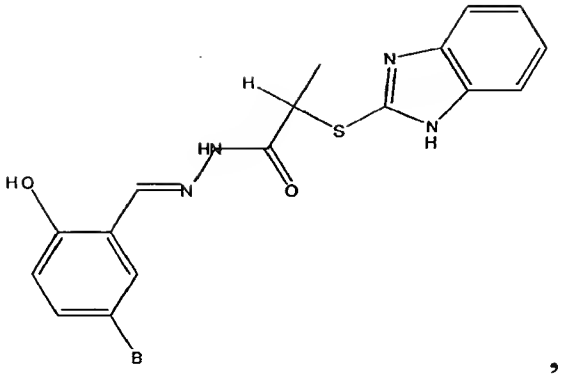
administering to the subject an agent that inhibits soluble adenylyl cyclase, said agent being selected from the group consisting of compounds having the following formulae:







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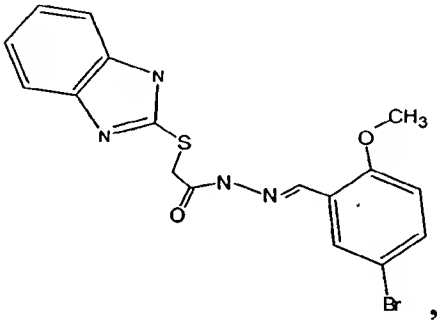
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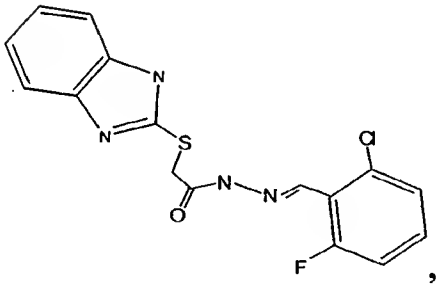
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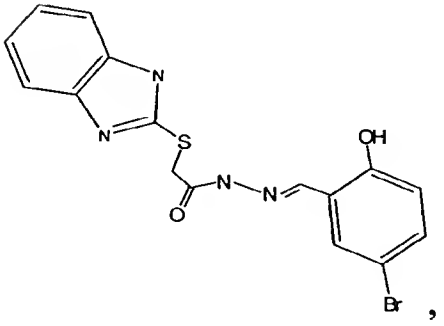
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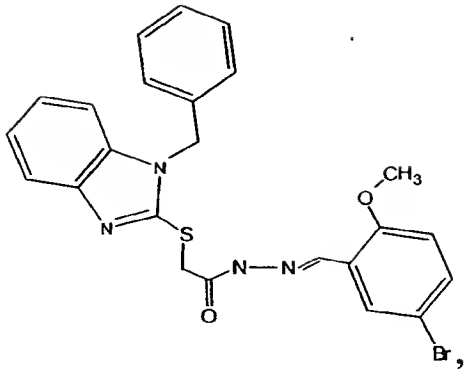

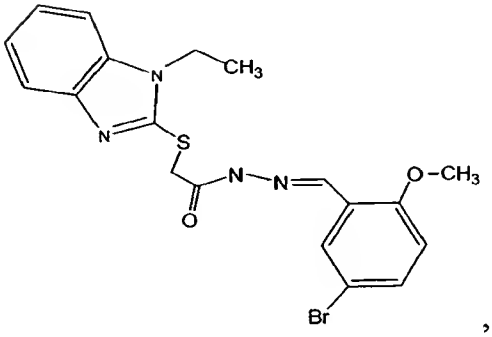

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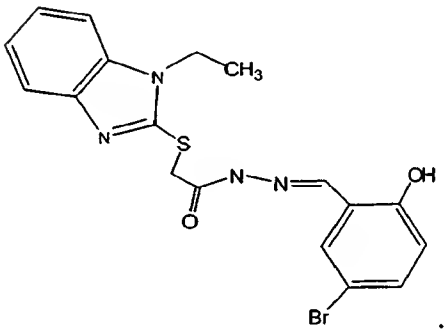
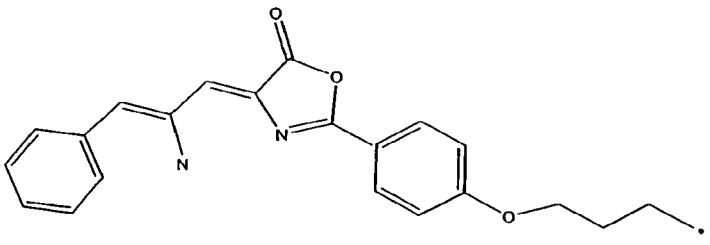
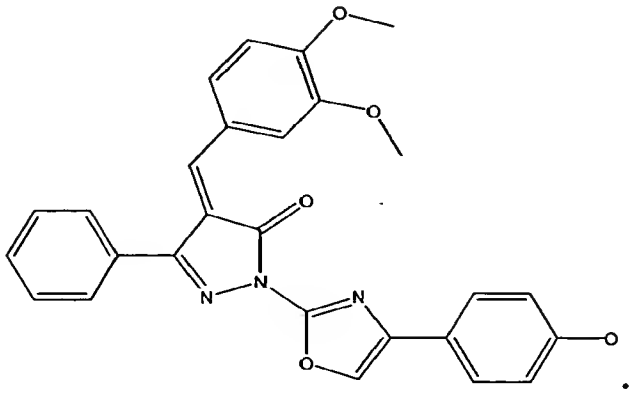


under conditions effective to treat the disorder mediated by soluble adenylyl cyclase.

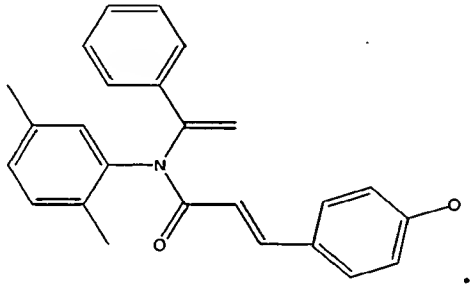
2. The method according to claim 1, wherein the compound has the formula:



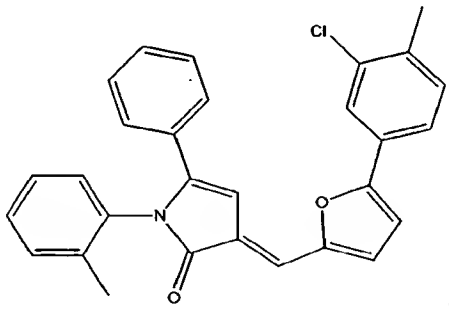
3. The method according to claim 1, wherein the compound has the formula:



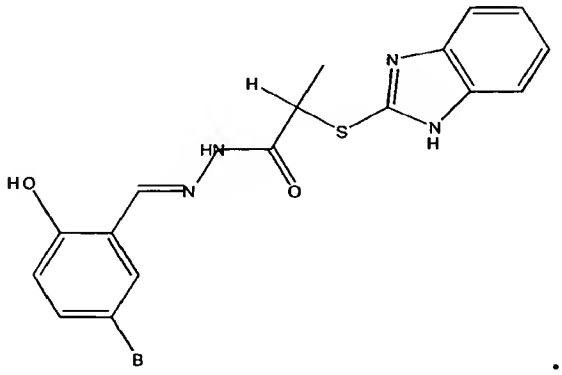
4. The method according to claim 1, wherein the compound has the formula:



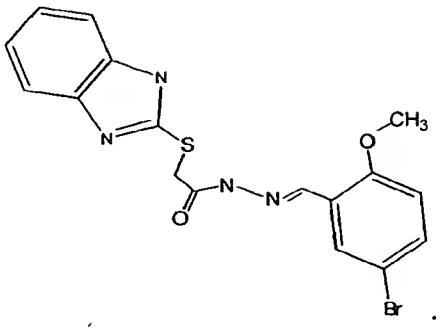
5. The method according to claim 1, wherein the compound has the formula:



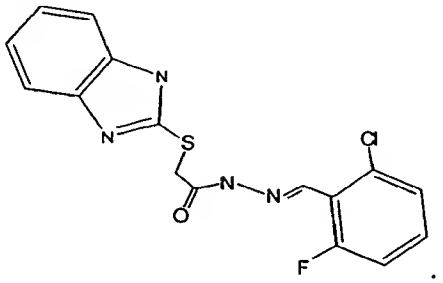
6. The method according to claim 1, wherein the compound has the formula:



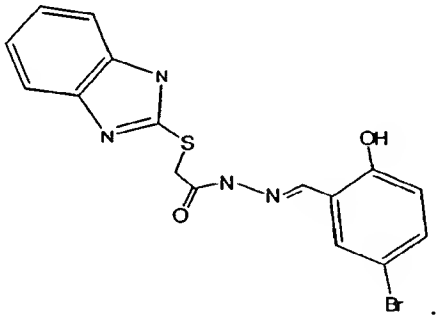
7. The method according to claim 1, wherein the compound has the formula:



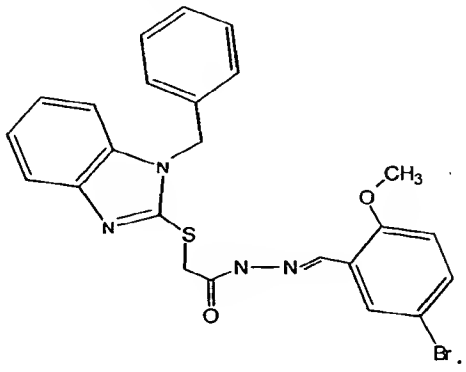
8. The method according to claim 1, wherein the compound has the formula:



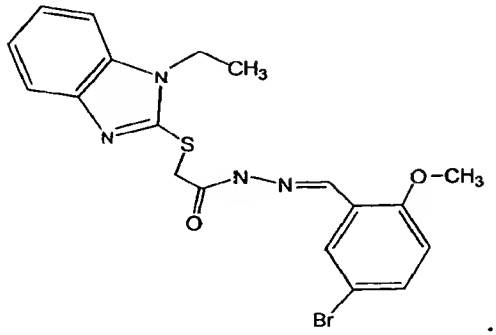
9. The method according to claim 1, wherein the compound has the formula:



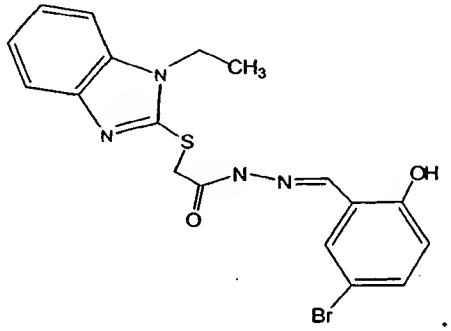
10. The method according to claim 1, wherein the compound has the formula:



11. The method according to claim 1, wherein the compound has the formula:



12. The method according to claim 1, wherein the compound has the formula:



13. The method according to claim 1, wherein the disorder is selected from the group consisting of learning or memory disorders, male fertility/sterility, glaucoma, metabolic acidosis/alkalosis, diabetes, metabolic disorders, breathing disorders, and insulin resistance.

14. The method according to claim 13, wherein the disorder is a learning or memory disorder.

15. The method according to claim 13, wherein the disorder is male fertility/sterility.

16. The method according to claim 13, wherein the disorder is glaucoma.

17. The method according to claim 13, wherein the disorder is metabolic acidosis/alkalosis.

18. The method according to claim 13, wherein the disorder is diabetes.

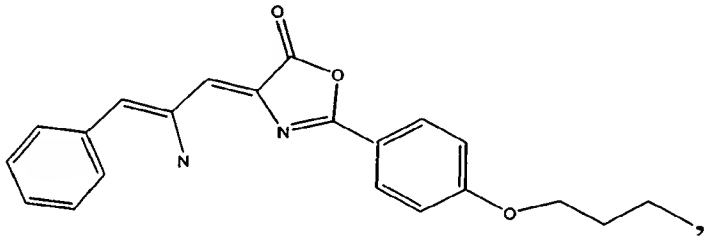
19. The method according to claim 13, wherein the disorder is a metabolic disorder.

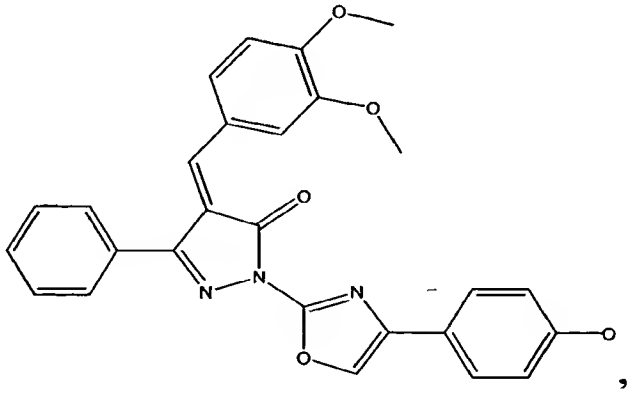
20. The method according to claim 13, wherein the disorder is a breathing disorder.

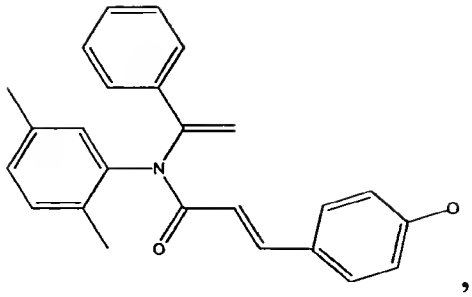
21. The method according to claim 13, wherein the disorder is an insulin resistance.

22. A method of inhibiting soluble adenylyl cyclase, said method comprising:

contacting eukaryotic cells with an agent that inhibits soluble adenylyl cyclase, said agent being selected from the group consisting of compounds having the following formulae:



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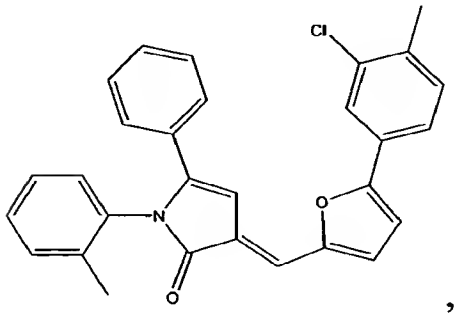
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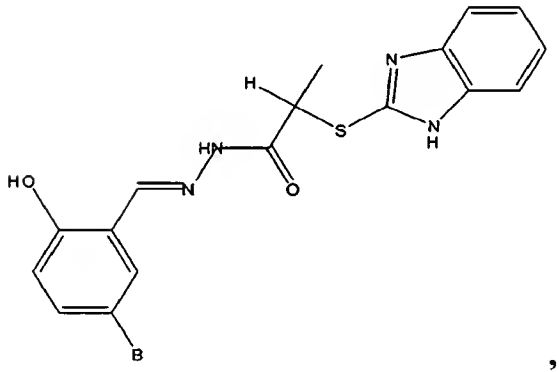
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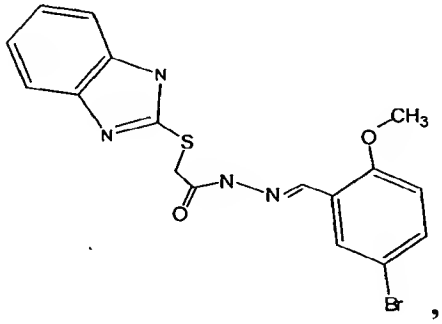
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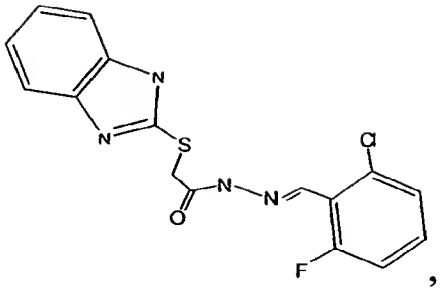
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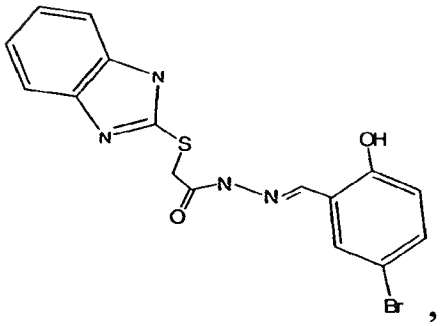
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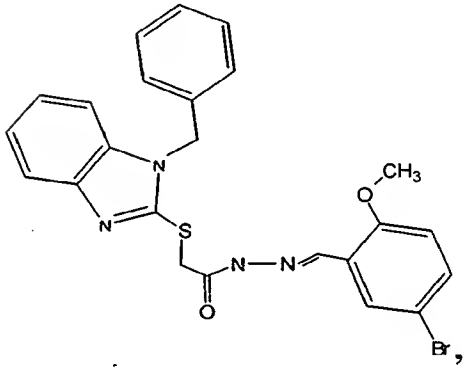
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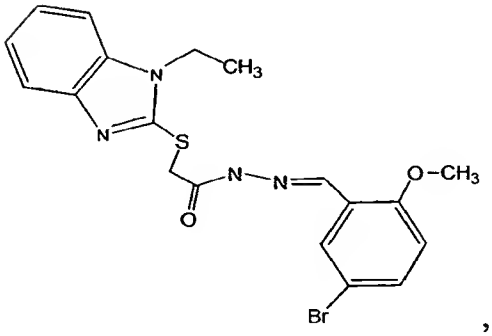
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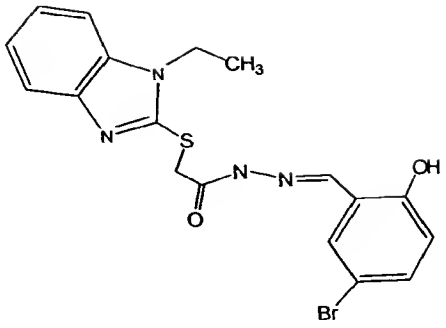
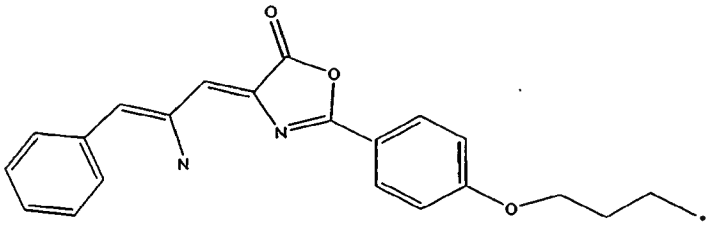
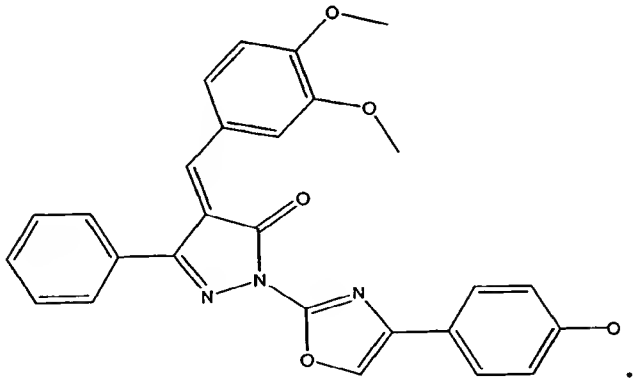


under conditions effective to inhibit soluble adenylyl cyclase.

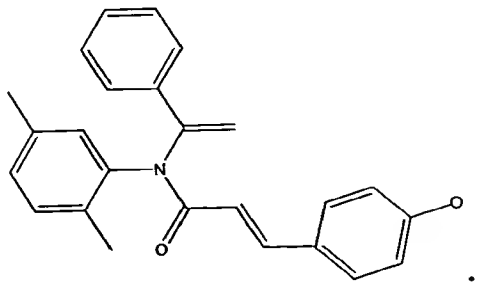
23. The method according to claim 22, wherein the compound has the formula:



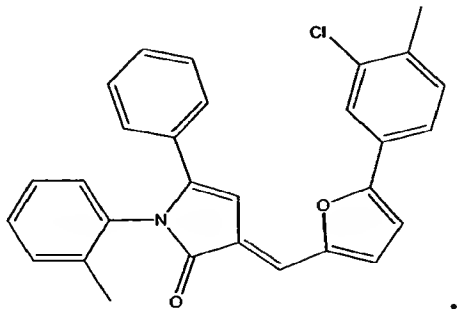
24. The method according to claim 22, wherein the compound has the formula:



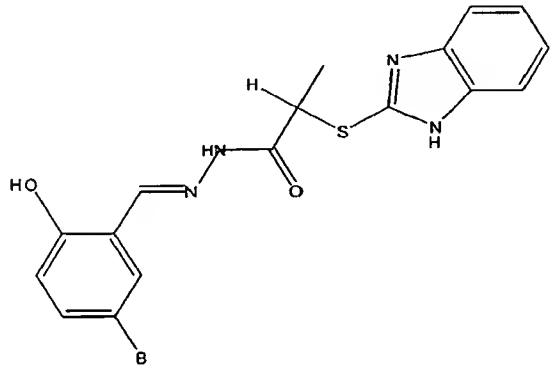
25. The method according to claim 22, wherein the compound has the formula:



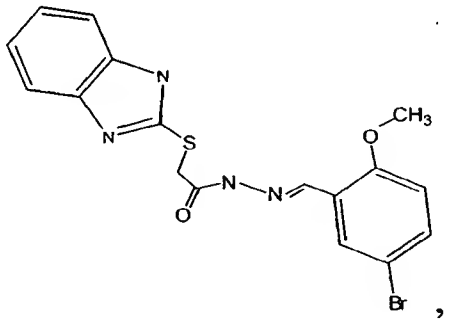
26. The method according to claim 22, wherein the compound has the formula:



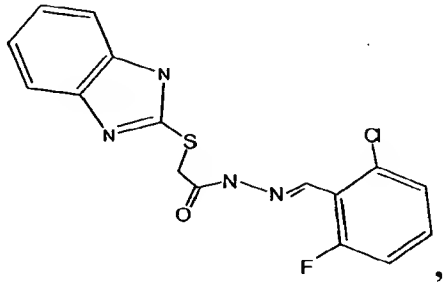
27. The method according to claim 22, wherein the compound has the formula:



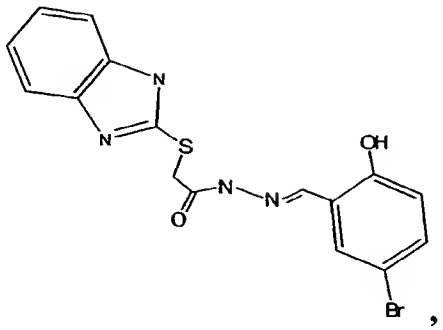
28. The method according to claim 22, wherein the compound has the formula:



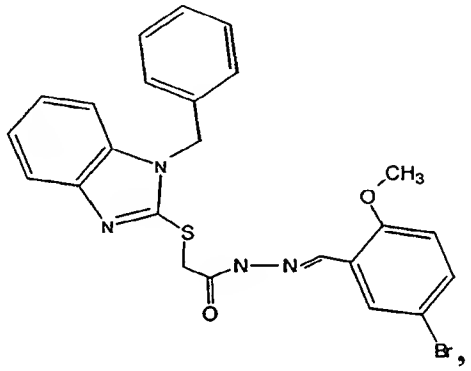
29. The method according to claim 22, wherein the compound has the formula:



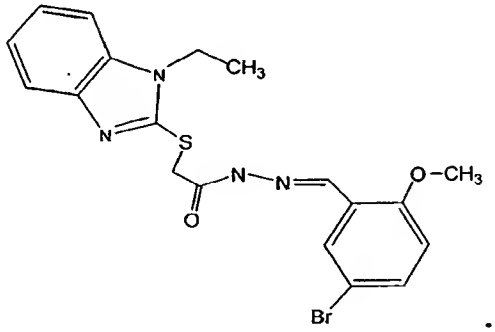
30. The method according to claim 22, wherein the compound has the formula:



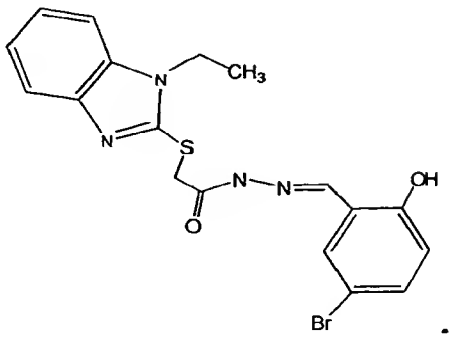
31. The method according to claim 22, wherein the compound has the formula:



32. The method according to claim 22, wherein the compound has the formula:



33. The method according to claim 22, wherein the compound has the formula:



Abstract

Bicarbonate responsive 'soluble' adenylyl cyclase resides, in part, inside the mammalian cell nucleus where it stimulates the activity of nuclear Protein Kinase A to phosphorylate the cAMP Response Element (CRE) Binding Protein. The existence of this complete and functional, nuclear-localized cAMP pathway establishes that cAMP signals in intracellular microdomains and identifies an alternate pathway leading to CRE Binding Protein activation.

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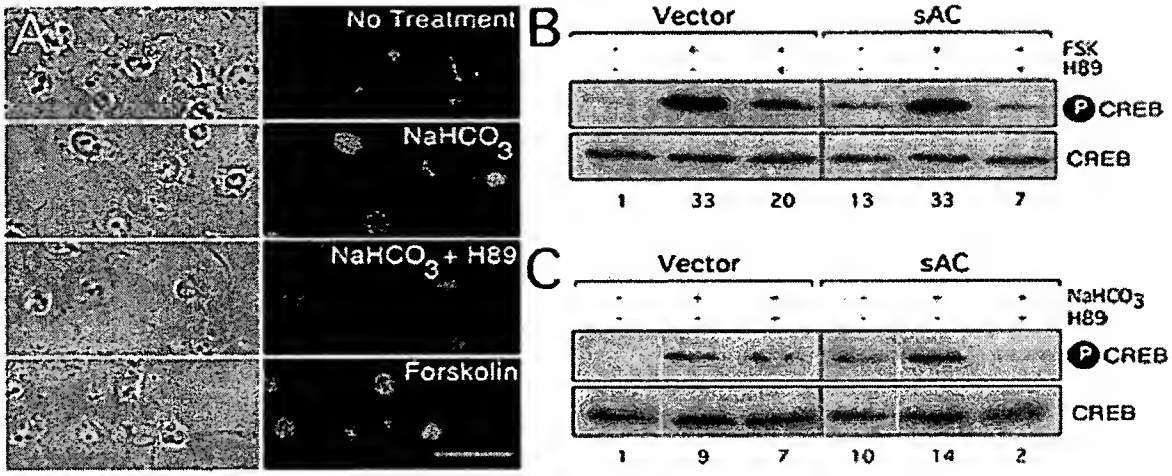
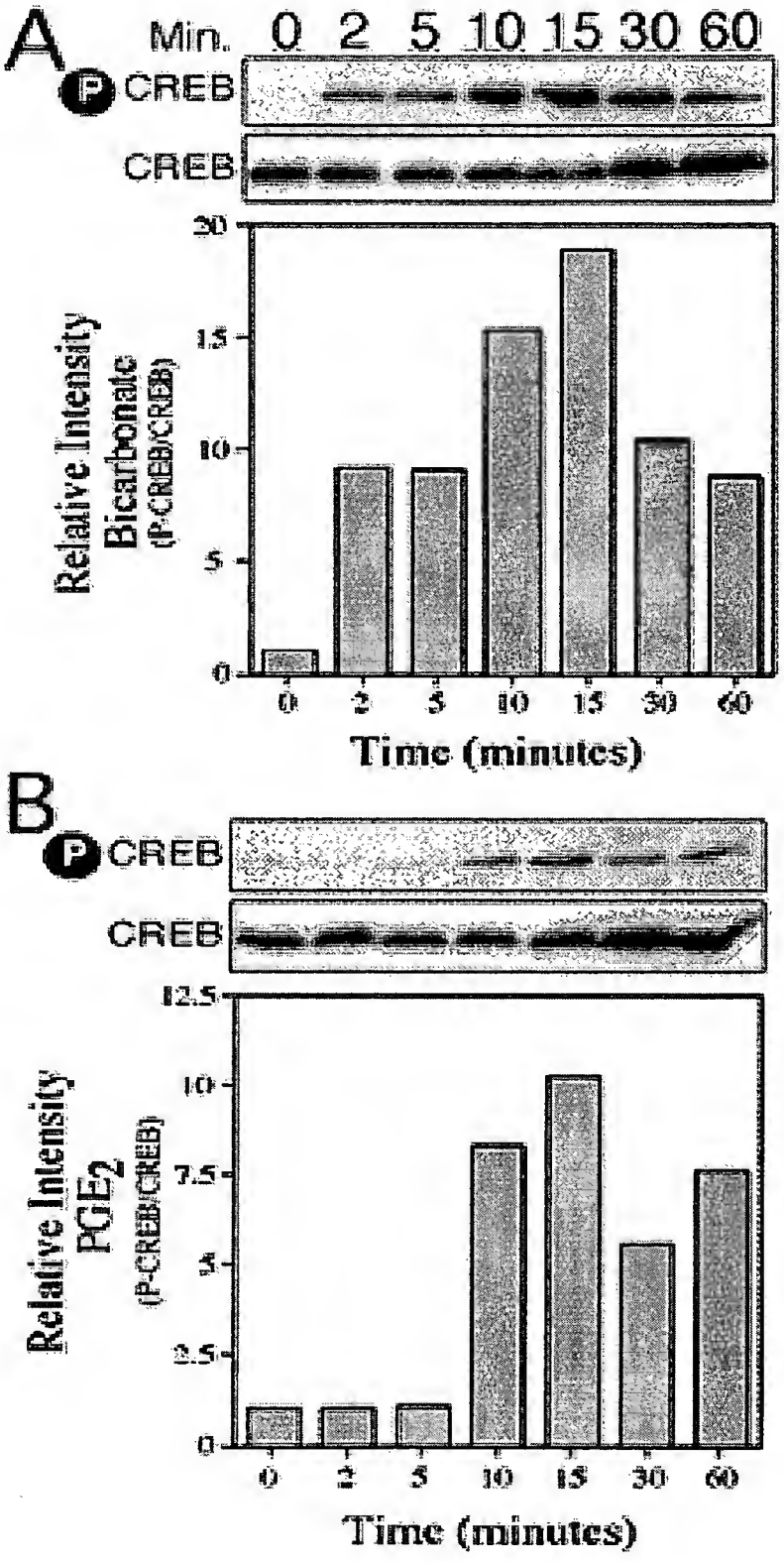
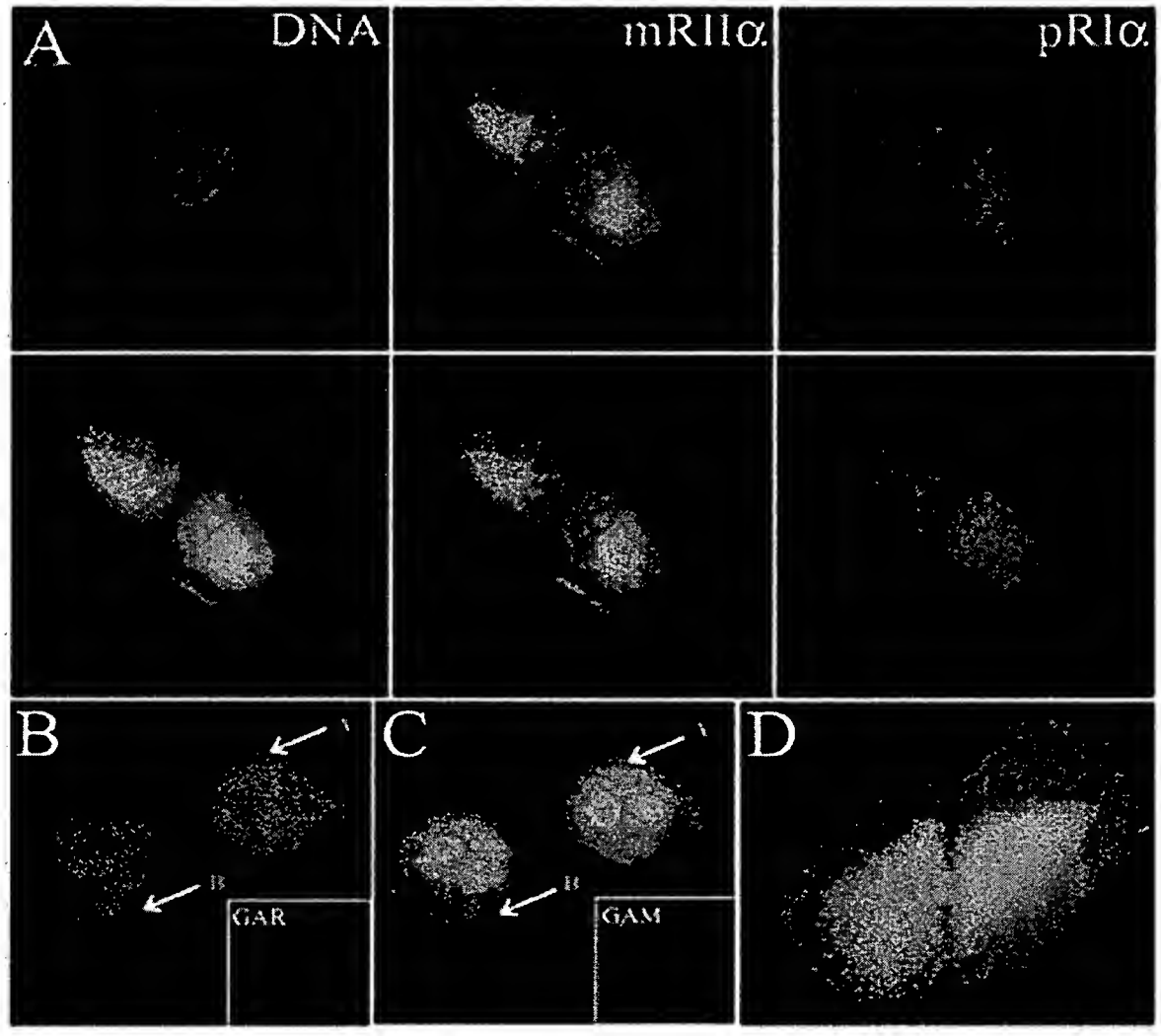
Zippin et al Figure 1



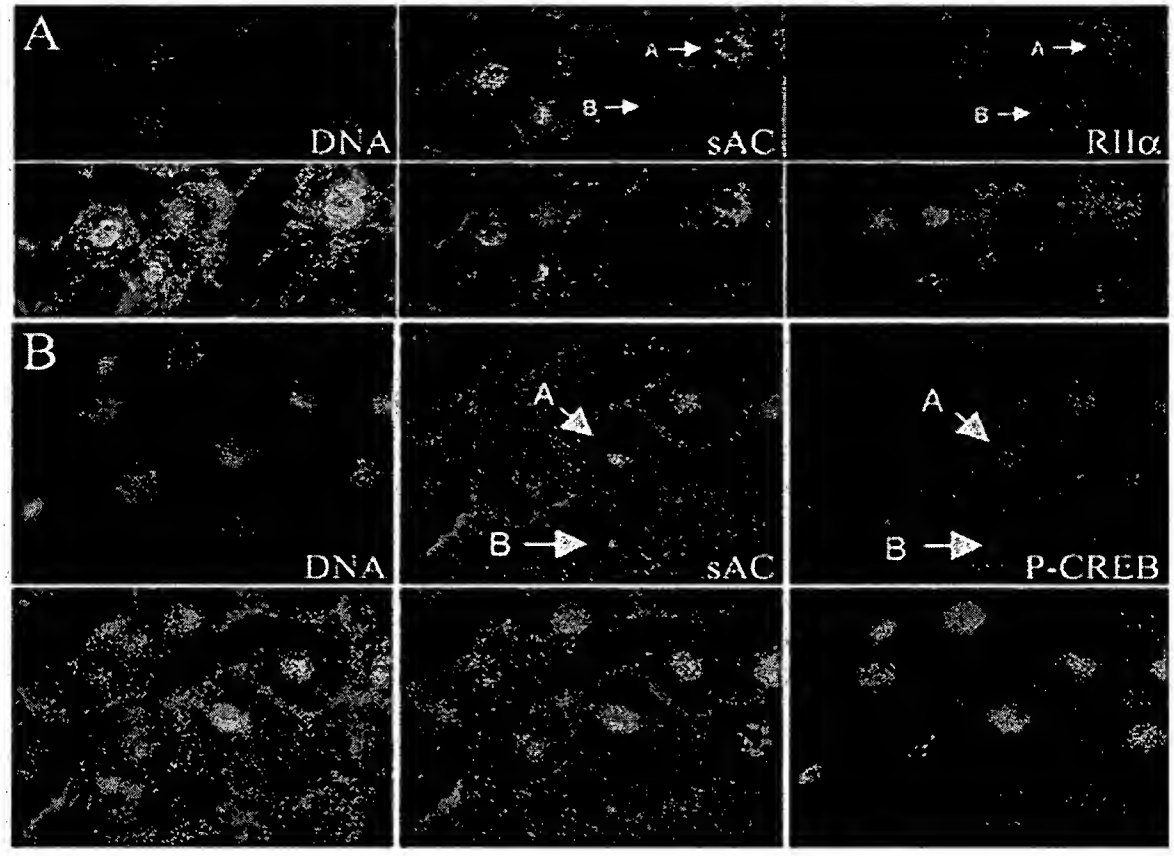
Zippin Figure 2



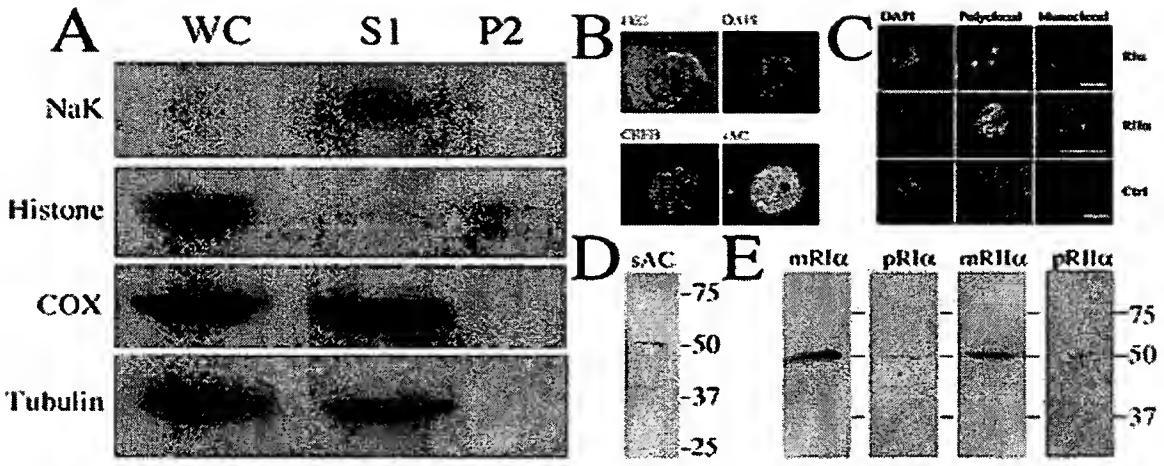
Zippin et al Figure 3



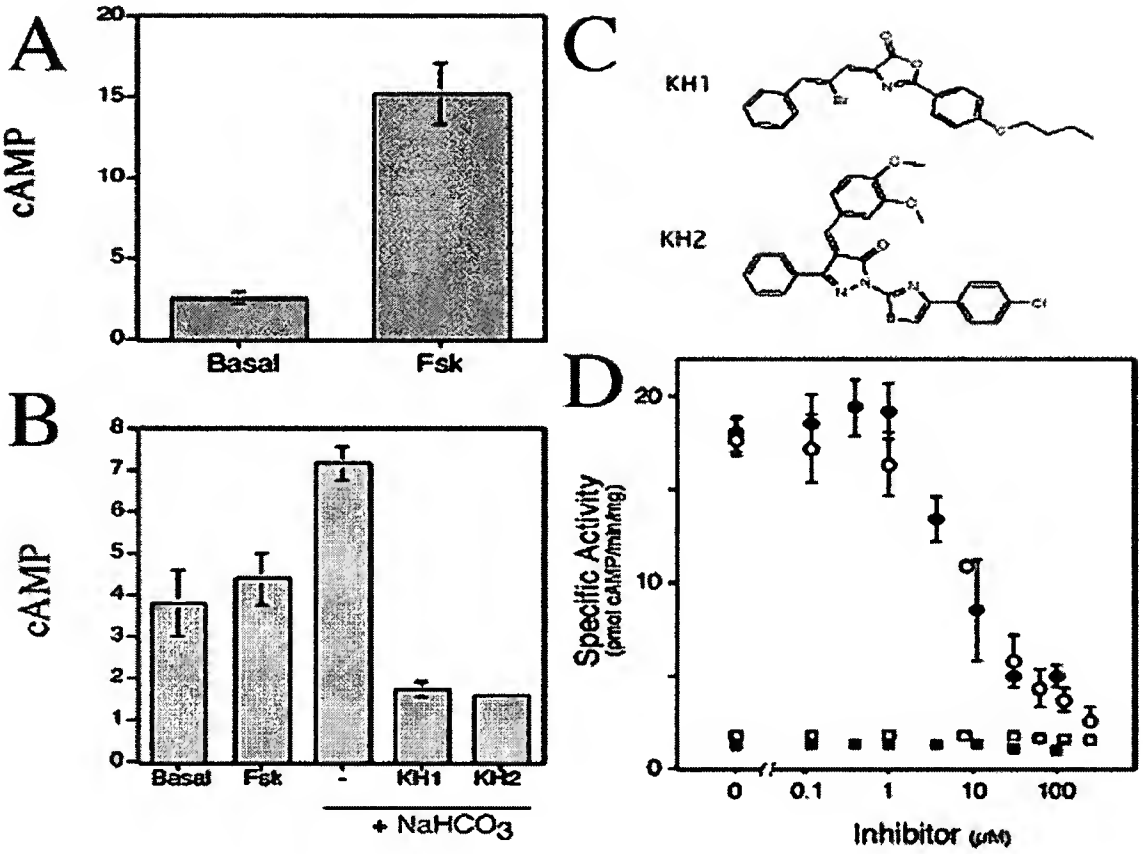
Zippin et al Figure 4



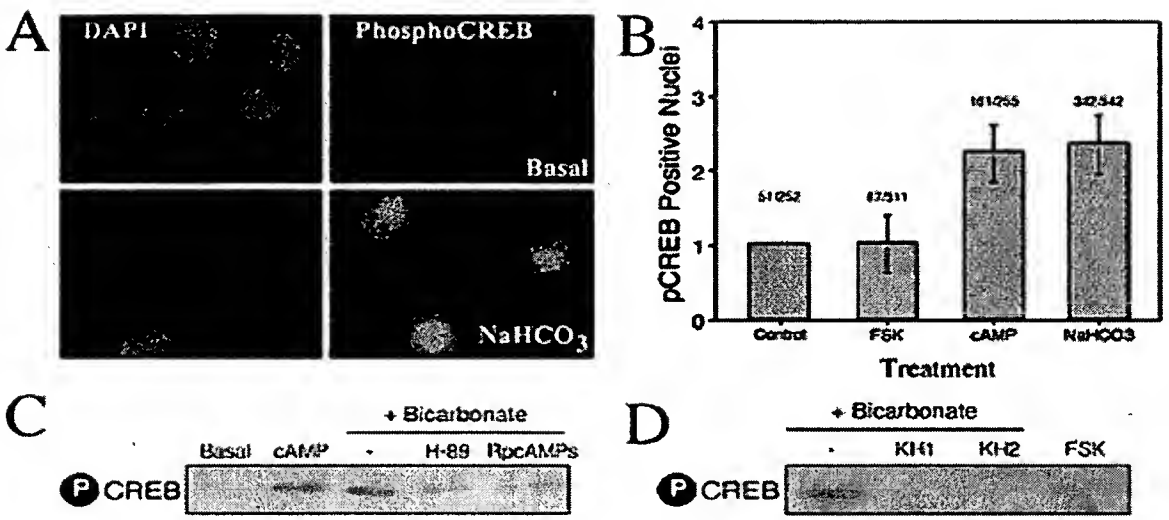
Zippin et al Figure 5



Zippin et al Figure 6



Zippin et al Figure 7



PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL984956509US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jochen	Buck	4 Ferris Drive Greenwich, CT 06870
Lonny R.	Levin	1161 York Avenue, Apt. 6D New York, NY 10021

☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

CHEMICAL INHIBITORS OF SOLUBLE ADENYLYL CYCLASE (sAC)

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

OR *Type Customer Number here*

☒ Firm *or* Individual Name	Michael L. Goldman				
Address	Nixon Peabody LLP				
Address	Clinton Square, P.O. Box 31051				
City	Rochester	State	NY	ZIP	14603-1051
Country	USA	Telephone	(585) 263-1304	Fax	(585) 263-1600

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 55 ☐ CD(s), Number [ ]

☐ Drawing(s) *Number of Sheets* [ ] ☐ Other (specify) [ ]

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge fees which may be required, or credit any overpayment to Deposit Account Number: 14-1138

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: National Institutes of Health Grant Nos. HD38722, GM62328, and HD42060.

Respectfully submitted, Date 1/21/04

SIGNATURE [signature]

TYPED or PRINTED NAME Michael L. Goldman

REGISTRATION NO. *(if appropriate)* 30,727

Docket Number: 19603/4650 (CRF D-3373-01)

TELEPHONE (585) 263-1304

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

R737627.1

The PTO did not receive the following listed item(s) 20 Sheets of claim

FEE TRANSMITTAL FOR FY 2003

Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80

Complete if Known	
Application Number	To Be Assigned
Filing Date	Herewith
First Named Inventor	Buck et al.
Examiner Name	
Art Unit	
Attorney Docket No.	19603/4650 (CRF D-3373-01)

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 14-1138

Deposit Account Name: Nixon Peabody LLP

The Commissioner is authorized to: *(check all that apply)*

☐ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s)

☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80

SUBTOTAL (1) ($) 80

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below		Fee Paid
Total Claims	-20** =		X		=	0
Independent Claims	-3** =		X		=	0
Multiple Dependent			X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive – unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0

CERTIFICATE OF MAILING OR TRANSMISSION [37 CFR 1.8(a)]

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Name *(Print/Type)*	Michael L. Goldman	Registration No. *(Attorney/Agent)*	30,727	Telephone	(585) 263-1304
Signature	Michael L. Goldman			Date	January 21, 2004

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EXPRESS MAIL CERTIFICATE

DOCKET NO.: **19603/4650 (CRF D-3373-01)**

APPLICANTS: **Jochen Buck and Lonny R. Levin**

TITLE: **CHEMICAL INHIBITORS OF SOLUBLE ADENYLYL CYCLASE (sAC)**

Certificate is attached to the **Provisional Application for Patent Cover Sheet (1 page) and Fee Transmittal (1 page)** of the above-identified application.

"EXPRESS MAIL" NUMBER: **EL984956509US**
DATE OF DEPOSIT: **January 21, 2004**

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Shawn A. Lockett
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(Signature of Person Mailing Paper or Fee)